PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 31/56 — A1

(11) International Publication Number: WO 95/17192

(43) International Publication Date: 29 June 1995 (29.06.95)

(21) International Application Number: PCT/US94/14624

(22) International Filing Date: 19 December 1994 (19.12.94)

(30) Priority Data:
08/173,243 22 December 1993 (22.12.93) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/173,243 (CIP)
Filed on 22 December 1993 (22.12.93)

(71) Applicant (*for all designated States except US*): THE SALK INSTITUTE FOR BIOLOGICAL STUDIES [US/US]; 10010 North Torrey Pines Road, La Jolla, CA 92037 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): COHN, Suzanne, Bourgeois [US/US]; 15151 Sun Valley Lane, Del Mar, CA 92014 (US). GRUOL, Donald, J. [US/US]; 2508 Vantage Way, Del Mar, CA 92014 (US).

(74) Agent: REITER, Stephen, E.; Pretty, Schroeder, Brueggemann & Clark, 444 South Flower Street, Los Angeles, CA 90071 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

AE

(54) Title: METHODS FOR REDUCING MULTIDRUG RESISTANCE

(57) Abstract

In accordance with the present invention, it has been discovered that certain steroid-like compounds are capable of inhibiting the efflux pump which is believed to be responsible for multidrug resistance. Thus, chemotherapy can be enhanced by facilitating the accumulation of drug at the target site, with reduced or eliminated competition by the drug efflux system.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

Methods for Reducing Multidrug Resistance

FIELD OF THE INVENTION

The present invention relates to methods for the treatment of multidrug resistance. In another aspect, the present invention relates to methods of enhancing the intracellular accumulation of molecules within a cell. In yet another aspect, the present invention relates to methods of enabling molecules to cross the blood-brain barrier.

BACKGROUND OF THE INVENTION

The treatment of human tumors with cytotoxic drugs is an important part of modern clinical cancer therapy. However, effective tumor treatment is frequently thwarted by the lack of sensitivity of certain tumors to standard chemotherapeutic agents (intrinsic resistance) or by the ability of certain tumors to develop chemotherapeutic resistance during the course of treatment (acquired resistance). The cause of this phenomenon has, at least in part, been demonstrated to result from the existence of an energy-dependent efflux pump which acts to remove the chemotherapeutic agent from the target cell.

The pump consists of P-glycoprotein found as a constituent of the cell membrane. It has been suggested that the normal function of P-glycoprotein is to remove toxins from within the cell. This theory is supported by the observation that P-glycoprotein is found as a cell membrane constituent in cells such as liver, kidney, colon, and jejunum. It has also been suggested that P-glycoprotein in the cell membrane of normal tissues could act to remove toxins or to assist in the transport of nutrients and solutes, and in secreting a variety of protein and steroid substances. The natural presence of P-glycoprotein in tumor cells derived from these tissues, as

well as its presence in tumor cells derived from other tissue types could explain, at least in part, resistance of various tumors to therapy with standard chemotherapeutic agents. Indeed, cancer cells demonstrate cross resistance to a diverse group of lipophilic drugs with unrelated structures and functions, a phenomenon known as multidrug resistance (MDR).

Drugs of proven antitumor chemotherapeutic value to which multidrug-resistance has been observed include vinblastine, vincristine, etoposide, teniposide, doxorubicin (adriamycin), daunorubicin, plicamycin (mithramycin), taxol and actinomycin D. Many tumors are intrinsically multidrug-resistant (e.g., adenocarcinomas of the colon and kidney) while other tumors acquire multidrug-resistance during therapy (e.g., neuroblastomas and childhood leukemias).

Several strategies have been devised to circumvent clinical MDR. One promising approach is the utilization of chemosensitizing agents which can inhibit active efflux of drugs in resistant cells. Numerous compounds including calcium antagonists, calmodulin inhibitors, and some drug analogues have shown variable abilities to reverse MDR. Most of these agents are lipophilic and may act as a ligand for the P-glycoprotein (i.e., bind to P-glycoprotein), thereby competitively inhibiting its drug efflux effect. Excellent reviews have recently been published on agents that alter multidrug resistance in cancer. See, for example, James M. Ford and William N. Hait, Pharmacology of Drugs that Alter Multidrug Resistance in Cancer, Pharmacological Reviews, Vol. 42:155-199 (1990); David J. Steward and William K. Evans, Non-Chemotherapeutic Agents that Potentiate Chemotherapy Efficacy, Cancer Treatment Reviews, Vol. 16:1-40 (1989).

The major factor thus far limiting the use of certain MDR reversing agents in cancer patients is the toxicity of such reversing agents, which prevents them from reaching effective concentrations during treatment. Another factor limiting the use of MDR reversing agents is the occurrence of undesired side effects caused by the agent employed. Thus, a substantial challenge remains in the search for ideal MDR reversing agents, i.e., agents which are pharmacologically acceptable for clinical applications, and which are more potent, but less toxic (and/or promote fewer side reactions) than reversing agents employed in the prior art.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, we have discovered that certain steroid-like compounds are capable of inhibiting the efflux pump which is believed to be responsible for multidrug resistance. Thus, chemotherapy can be enhanced by facilitating the delivery of drug to the target site, with reduced or eliminated competition by the drug efflux system. Compounds employed in the practice of the present invention inhibit the drug efflux pump, but do not have substantial affinity for steroid receptors. Thus, invention compounds do not exhibit the hormonal side effects observed with such prior art reversing agents as verapamil or progesterone.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the reversal of puromycin resistance in S7CD-5 cells by RU486 and verapamil. Incubation of S7CD-5 cells with no drug addition is designated by O; incubation with 5μM RU486 is designated by $\Delta$and incubation with 5μM verapamil is designated by $\blacktriangle$. Incubation of parental cell line W7TB (from which S7CD-5 is derived) is designated $\bullet$.

Figure 2 illustrates the reversal of daunomycin resistance in S7CD-5 cells by RU486 and verapamil. The legend for the curves presented in this figure is the same as the legend employed in Figure 1.

Figure 3 illustrates the effect of verapamil and various concentrations of RU486 on the efflux of rhodamine 123 from S7CD-5 cells. The control is identified by ○, verapamil (5μM) is identified by △, RU486 (10μM) is identified by ●, RU486 (5μM) is identified as ♦, and RU486 (1μM) is identified as ■.

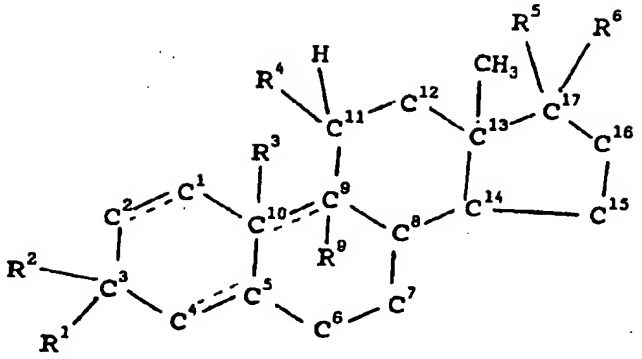
Figure 4 illustrates the effect of dexamethasone, progesterone, and RU486 on the efflux of rhodamine 123 from S7CD-5 cells. The control is identified by ○, dexamethasone is identified by △, progesterone is identified by □, and RU486 is identified as ●.

Figure 5 illustrates the effect of various agents on the sensitivity of a human leukemic cell line to exposure to vinblastine. Incubation of ~$5x10^4$ cells/ml of a human leukemic cell line selected for resistance to vinblastine with no other drug addition is designated by □; incubation with 5μM verapamil is designated by ▲; incubation with 5μM RU486 is designated by ■; and Incubation with 5μM progesterone is designated by △.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided a method for reducing multidrug resistance in a subject undergoing chemotherapy, said method comprising:

administering to said subject, in conjunction with a chemotherapeutic agent, an effective amount of a compound having the structure:



wherein:

$R^1 = R^2 = O$; or R^1 = hydrogen and

R^2 is OR, wherein R is selected from hydrogen, lower alkyl or trimethylsilyl; and

R^3 is absent when there is a double bond between C^9 and C^{10}, or when there is an epoxide bridging C^9 and C^{10}, or R^3 is hydrogen or methyl; and

R^4 is -OR', wherein R' is lower alkyl or trimethylsilyl, or R^4 is an organic radical having in the range of 4 up to 18 carbon atoms containing at least one atom selected from the group consisting of oxygen, nitrogen, phosphorus and silicon, wherein the atom immediately adjacent C^{11} is carbon, and said organic radical includes a cyclic moiety selected from an alicyclic ring, a heterocyclic ring, a carbocyclic aromatic ring, or a heterocyclic aromatic ring, wherein said cyclic moiety contains an oxygen-, nitrogen-, phosphorus- or silicon-bearing substituent,

R^5 is hydrogen or OR, wherein R is as defined above, or R^5 is a 3, 4 or 5 atom bridging species which forms part of a 3-, 5-, 6- or 7-membered ring including C^{16} and C^{17} as part of the ring; and

R^6 is selected from:

$-C(O)-CH_3$,

$-CH(OH)-CH_3$,

$-C(O)-CH_2OH$, or

$-(R^7)_{0,1}-R^8$, wherein

R^7, when present, is a saturated or unsaturated (straight or branched chain) hydrocarbyl radical having in the range of 1 up to 8 carbon atoms, and

R^8 is selected from hydrogen, halogen, trimethylsilyl, phenyl or substituted phenyl, and

R^9 is absent when there is a double bond between C^9 and C^{10}, or, when present, R^9 is selected from hydrogen or halogen.

Optionally, compounds employed in the practice of the present invention can have double bond(s) in the steroid "A" ring between C^1 and C^2 and/or between C^4 and C^5, and/or a double bond in the steroid "B" ring between C^9 and C^{10}. Those of skill in the art recognize that substituent "R^3" will not be present when there is a double bond in the "B" ring. As an alternative to a carbon-carbon double bond between C^9 and C^{10}, an epoxide can also bridge these two carbons. Compounds containing an epoxide bridging carbons C^9 and C^{10} can optionally also have a double bond in the steroid "A" ring between C^4 and C^5.

In a presently preferred aspect of the present invention, R^4 is defined by the following general formula:

$$-(CH_2)_x-(C_6H_{3,4})-[X_{0,1}-(CH_2)_y-X']_z;$$

wherein:

x is 0-3 (preferably 0),

y is 0-2,

z is 1 when the aromatic ring is C_6H_4, or

2 when the aromatic ring is C_6H_3,

X is O or S,

X' is hydrogen, OR, $NR"_2$, $N^+R"_3$, $\overset{O^-}{\underset{|}{N^+}}R"_2$, or NO_2, wherein R is as defined above and R" is hydrogen or a lower alkyl group.

When z of the above general formula is 1, the substituent on the phenyl ring is preferably located at the para position of the ring. Especially preferred para substituents include:

$-O-CH_2CH_2-NR"_2$, $-O-CH_2CH_2-N^+R"_3$,

$-O-CH_2CH_2-\overset{O^-}{\underset{|}{N^+}}R"_2$, and the like.

When z of the above general formula is 2, the substituents on the phenyl ring are preferably located at the meta and para positions of the ring, wherein the preferred para substituents are selected from OR, $NR"_2$, $N^+R"_3$, $\overset{O^-}{\underset{|}{N^+}}R"_2$, or NO_2.

When R^5 is a 3, 4 or 5 atom bridging species which forms part of a 3-, 5-, 6- or 7-membered ring including C^{16} and C^{17} as part of the ring, R^5 is typically selected from straight or branched chain alkylene, or O-, C(O)-, N-, and/or S-containing alkylene moieties, such as, for example, $-(CH_2)_{3-5}-$, $-C(CH_3)_2-$ (i.e., isopropylidene), $-O-(CH_2)_{2-4}-$, $-O-(CH_2)_{1-3}-O-$, $-(CH_2)_{1,2}-O-(CH_2)_{1,2}-$, $-O-CH_2-C(O)-CH_2-O-$ (i.e., acetonide), and the like.

In a presently preferred aspect of the present invention, R^6 is selected from:

$-C(O)-CH_3$,

$-CH(OH)-CH_3$, or

$-C(O)-CH_2OH$, or

R^6 is defined by the following general formulae:

$-(CH_2)_{0,1}-C=C-R^8$, or

$-(CH_2)_{0,1}-C\equiv C-R^8$, wherein

R^8 is selected from hydrogen, chloro, trimethylsilyl or phenyl.

Presently preferred structures for R^6 include:

$-CH_2-CH=CH_2$,

$-CH_2-C\equiv CH$,

$-C\equiv C-H$,

$-C\equiv C-CH_3$,

$-C\equiv C-Cl$,

$-C\equiv C-C_6H_5$, or

$-C\equiv C-SiMe_3$.

R^7 is preferably a straight chain alkylene, or straight chain alkynylene moiety having the structure $-(CH_2)_x-CR'=CR'-(CH_2)_x-$ or $-(CH_2)_x-C\equiv C-(CH_2)_x-$, wherein each x is independently selected from 0-6.

In a presently preferred aspect of the present invention, R^8 is selected from hydrogen, chloro, trimethylsilyl or phenyl, and R^9, when present, is hydrogen.

Presently preferred compounds of the invention are those where:

$R^1 = R^2 = O$ (i.e., a carbonyl at C^3),

R^3 is methyl,

R^4 is

$-(CH_2)_x-(C_6H_{3,4})-[X_{0,1}-(CH_2)_y-X']_z$;

wherein:

x is 0-3 (with x = 0 presently preferred),

y is 0-2,

z is 1 when the aromatic ring is C_6H_4, or
2 when the aromatic ring is C_6H_3,

X is O or S,

X' is hydrogen, OR, $NR"_2$, $N^+R"_3$, $N^+R"_2O^-$, or NO_2, wherein R and R" are as defined above,

R^5 is hydrogen,

R^6 is selected from

$-C(O)-CH_3$,

$-CH(OH)-CH_3$,

$-C(O)-CH_2OH$, or

R^7 is selected from

$-(CH_2)_x-CR"=CR"-(CH_2)_x-R^8$ or

$-(CH_2)_x)-C\equiv C-(CH_2)_x-R^8$,

wherein each R" is as defined above, and each R" is selected independently of each other, and x is independently 0-6, and

R^8 is selected from hydrogen, chloro, trimethylsilyl or phenyl, and

R^9 is hydrogen.

Where the phenyl group of R^4 is mono-substituted, it is preferred that the substituent be located at the para position of the ring. When the phenyl group of R^4 is di-substituted, it is preferred that the substituents be located at the meta and para positions of the ring.

In especially preferred embodiments of the present invention, R^4 is selected from:

$-C_6H_4-p-O-CH_2CH_2-NR"_2$,

$-C_6H_4-p-O-CH_2CH_2-N^+R"_3$,

$-C_6H_4-p-O-CH_2CH_2-N^+R"_2O^-$, or

$-C_6H_3-m-OCH_3$, $p-OCH_3$,

and R^6 is selected from:

$-C(O)-CH_3$,

$-CH(OH)-CH_3$,

$-C(O)-CH_2OH$,
$-CH_2-CH=CH_2$,
$-CH_2-C\equiv CH$,
$-C\equiv C-H$,
$-C\equiv C-CH_3$,
$-C\equiv C-Cl$,
$-C\equiv C-C_6H_5$, or
$-C\equiv C-SiMe_3$.

Compounds contemplated for use in the practice of the present invention can be readily prepared using techniques which are well known in the art. See, for example, Teutsch et al., in J. Steroid biochemistry 31:549-565 (1988) and United States Patent No. 4,386,085, the entire contents of which are hereby incorporated by reference herein.

The compounds of the present invention are capable of enhancing the sensitivity of multidrug resistant tumor cells to antitumor chemotherapeutic agents. In addition, the compounds of the present invention are useful in preventing the emergence of multidrug resistant tumor cells during a course of treatment with antitumor chemotherapeutic agents. The compounds of the present invention are further useful in reducing the effective dosage of chemotherapeutic agent required during treatment of multidrug resistant tumors.

Clinical multidrug resistance can develop in response to a number of important chemotherapeutic agents, including vinblastine (0.1 mg per kilogram per week), vincristine (0.01 mg per kilogram per week), dactinomycin (0.015 mg per kilogram per day), daunorubicin (60 to 75 mg per square meter per week), doxorubicin (500 to 600 mg per square meter), etoposide (35 to 50 mg per square meter per day), and mithramycin (0.025 mg per kilogram per day).

Multidrug resistance has been shown to occur *in vitro* as well as in the clinic.

Multidrug resistant cell lines are easily developed for *in vitro* determination of the ability of compounds of the present invention to reduce multidrug resistance. Such cell lines can be readily developed in accordance with the methods described by Twentyman et al., Br. J. Cancer, Vol. 54:253 (1986). The Twentyman procedure selects for multidrug resistant cells by culturing the parental drug sensitive cell line in the continued presence of a cytotoxic drug, for example, doxorubicin.

Drug sensitive cells will perish because the ability of the efflux pump to prevent accumulation of drug into the cell is reduced by the presence of compounds of the invention. In contrast, multidrug resistant cells will survive and grow despite the presence of the drug. Eventually, a multidrug resistant cell population emerges and can be used in an assay system for the detection of agents which can modify the multidrug resistance. Many cell lines are suitable as parental cell lines from which multidrug resistant cells can be selected. These cell lines can be derived from humans or other mammals and can be derived from normal tissue or tumor tissue. Commercially available human cell lines derived from human tumor tissue include KB (ATCC CCL 17), NCI-H69 (ATCC HTB 119), CCRF-CCM (ATCC CCL 119), and K-562 (ATCC CCL 243). Other suitable, commercially available mammalian cell lines include LM(TK-) (ATCC CCL 1.3), and CHO-K1 (ATCC CCL 61).

The sensitivity of drug resistant cell lines to chemotherapeutic agents can be compared with the parental cell line by assaying inhibition of cell growth during continuous exposure to the drug. Growth of the parental cells will be inhibited by the chemotherapeutic agent, while the growth of resistant cells will not be inhibited.

Cell growth can be measured by cell counting using an electronic cell counter, for example, a Coulter Counter, Coulter Electronics, Herts, England, and following the manufacturers recommended instructions for use. Cells may also be counted microscopically using a hemocytometer. The presently preferred technique is to combine the use of the hemocytometer with a stain (e.g., tryptan blue) that allows one to distinguish between living cells and dead cells.

Cell growth can also be measured by other techniques including cell staining. Cells can be stained by various agents including crystal violet, coomassie blue and methylene blue, with methylene blue being the presently preferred stain. Determining cell growth by methylene blue staining can be done as described in Example 1, below.

Cell growth measured by either the cell counting method or the cell staining method should closely correlate. The staining method is preferable because of its simplicity and it is easily adaptable to automation which allows many experiments to be performed with many test compounds non-labor intensively.

Radiolabelled compounds may also be utilized to determine the accumulation of antitumor chemotherapeutic agents in drug sensitive cells and in multidrug resistant cells. For example, the accumulation of [^{3}H]vinblastine by drug sensitive cell lines and drug resistant cell lines in the presence or absence of a compound of the invention may be determined. The relative accumulation of the radiolabelled chemotherapeutic agent is indicative of the ability of a compound of the invention to reduce multidrug resistance.

The modulation of multidrug resistance demonstrated by the compounds described herein provides a method for treatment of multidrug resistant tumors. The

multidrug resistant modulating properties of the compounds described herein also provide a method for preventing the emergence of multidrug resistant tumors during the course of cancer treatment. These same compounds additionally provide a method for reducing the required dosage of an antitumor chemotherapeutic agent.

All of the methods of this invention involve (1) the administration of a compound of the present invention, prior to or concurrent to the administration of an chemotherapeutic agent: or (2) the administration of a combination of one or more of the compounds of the present invention, and an antitumor chemotherapeutic agent.

Reference to administering compounds contemplated for use in the practice of the present invention "in conjunction with" a chemotherapeutic agent or "concurrently administering," compounds according to the present invention, as used herein, means that the antineoplastic agent and the agent employed for the reduction of multidrug resistance are administered either (a) simultaneously in time (optionally by formulating the two together in a common carrier), or (b) at different times during the course of a common treatment schedule. In the latter case, the two agents are administered at times sufficiently close for the agent employed for reducing multidrug resistance to enhance the selective growth-inhibiting action of the antineoplastic agent on the tumor cells.

For the treatment of multidrug resistant tumor cells the compounds of the present invention, either separately or in combination with a chemotherapeutic agent, may be administered orally, parenterally (including subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques) or rectally, in dosage unit formulations containing conventional non-

toxic, pharmaceutically-acceptable carriers, adjuvants and vehicles.

In particular, the compounds of the present invention may be administered either separately or in combination with an appropriate chemotherapeutic agent such as vinblastine, vincristine, dactinomycin, daunorubicin, doxorubicin, etoposide or mithramycin.

The precise mode of administration employed for each subject is left to the discretion of the practitioner. Because the compounds of the present invention exhibit appreciable oral activity, compounds of this invention can readily be administered orally. Alternatively, parenteral administration may be preferable in certain circumstances.

The compounds of the present invention are most easily administered in the form of a pharmaceutically acceptable non-toxic acid addition salt formed from an invention compound and an organic or inorganic acid recognized in the art as providing a pharmaceutically acceptable non-toxic acid addition salt. Examples of such acid addition salts include acetate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, dihydrogen phosphate, dodecylsulfate, ethanesulfonate, fumarate, hydrochloride, hydrobromide, hydroiodide, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, propionate, succinate, sulfate, tartrate, thiocyanate, tosylate, maleate, fumarate, or the like. A preferred embodiment of the invention is that in which a compound is present as the hydrochloride salt.

The compounds contemplated for the use in the practice of the present invention possess activity in increasing the sensitivity of multidrug resistant mammalian cells to chemotherapeutic agents in culture and are useful

in the treatment of multidrug resistant tumors in mammalian subjects.

The term "subject" used herein is taken to mean mammals such as primates, including humans, sheep, horses, cattle, pigs, dogs, cats, rats and mice.

The pharmaceutical compositions of this invention can be used in solid, semisolid or liquid form, which composition contains one or more of the compounds of the present invention, as an active ingredient, in admixture with an organic or inorganic carrier or excipient suitable for enteral or parenteral applications. The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use. The carriers which can be used include water, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea and other carriers suitable for use in manufacturing preparations, in solid, semisolid, or liquid form. In addition auxiliary, stabilizing, thickening and coloring agents and perfumes may be used. The active compound (i.e., the above-described multidrug resistance-reducing compounds) is included in the pharmaceutical composition in an amount sufficient to produce the desired effect upon the process or condition of diseases.

The pharmaceutical compositions containing the active ingredient may be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsions, hard or soft capsules, or syrups or elixirs. Compositions intended for oral use may be prepared according to any method known to the art for the manufacture of

pharmaceutical compositions and such compositions may contain one or more agents selected from the group consisting of a sweetening agent such as sucrose, lactose, or saccharin, flavoring agents such as peppermint, oil of wintergreen or cherry, coloring agents and preserving agents in order to provide pharmaceutically elegant and palatable preparations. Tablets containing the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients may also be manufactured by known methods. The excipients used may be, for example, (1) inert diluents such as calcium carbonate, lactose, calcium phosphate or sodium phosphate; (2) granulating and disintegrating agents such as corn starch, potato starch or alginic acid; (3) binding agents such as gum tragacanth, corn starch, gelatin or acacia, and (4) lubricating agents such as magnesium stearate, stearic acid or talc. The tablets may be uncoated or they may be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. for example, a time delay material such as glyceryl monostearate or glyceryl distearate may be employed. They may also be coated by the techniques described in the U.S. Pat. Nos. 4,256,108; 4,160,452; and 4,265,874, to form osmotic therapeutic tablets for controlled release.

In some cases, formulations for oral use may be in the form of hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin. They may also be in the form of soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example, peanut oil, liquid paraffin, or olive oil.

Aqueous suspensions normally contain the active materials in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients may be

(1) suspending agents such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcelluose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia;

(2) dispersing or wetting agents which may be
(a) a naturally-occurring phosphatide such as lecithin,
(b) a condensation product of an alkylene oxide with a fatty acid, for example, polyoxyethylene stearate,
(c) a condensation product of an ethylene oxide with a long chain aliphatic alcohol, for example, heptadecaethyleneoxycetanol,
(d) a condensation product of ethylene oxide with a partial ester derived from a fatty acid and a hexitol such as polyoxyethylene sorbital monooleate, or
(e) a condensation product of ethylene oxide with a partial ester derived from a fatty acid and a hexitol anhydride, for example, polyoxyethylene sorbitan monooleate.

The aqueous suspensions may also contain one or more preservatives, for example, ethyl or n-propyl p-hydroxybenzoate; one or more coloring agents; one or more flavoring agents; and one or more sweetening agents such as sucrose or saccharine.

Oily suspension may be formulated by suspending the active ingredient in a vegetable oil, for example, arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions may contain a thickening agent, for example, beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents may be added to provide a palatable oral preparation. These compositions may be prepared by the addition of an antioxidant such as ascorbic acid.

Dispersible powders and granules are suitable for the preparation of an aqueous suspension. They provide the active ingredient in admixture with a dispersing or setting agent, a suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above. Additional excipients, for example, those sweetening, flavoring and coloring agents described above may also be present.

The pharmaceutical compositions of the invention may also be in the form of oil-in-water emulsions. The oily phase may be a vegetable oil such as olive oil or arachis oils, or a mineral oil such as liquid paraffin or a mixture thereof. Suitable emulsifying agents may be (1) naturally occurring gums such as gum acacia and gum tragacanth, (2) naturally occurring phosphatides such as soy bean and lecithin, (3) esters or partial esters derived from fatty acids and hexitol anhydrides, for example, sorbitan monooleate, (4) condensation products of said partial esters with ethylene oxide, for example, polyoxyethylene sorbitan monooleate. The emulsions may also contain sweetening and flavoring agents.

Syrups and elixirs may be formulated with sweetening agents, for example, glycerol, propylene glycol, sorbitol or sucrose. Such formulations may also contain a

demulcent, a preservative such as methyl and propyl parabans, flavoring such as cherry or orange flavor and coloring agents.

The pharmaceutical compositions may be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to known methods using those suitable dispersing or wetting agents and suspending agents which have been mentioned above. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides, fatty acids (including oleic acid), naturally occurring vegetable oils like sesame oil, coconut oil, peanut oil, cottonseed oil, etc., or synthetic fatty vehicles like ethyl oleate or the like. Buffers, preservatives antioxidants and the like can be incorporated as required.

Compounds contemplated for use in the practice of the present invention may also be administered in the form of suppositories for rectal administration of the drug. These compositions may be prepared by mixing the drug with a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters of polyethylene glycols, which are solid at ordinary temperatures, but liquify and/or dissolve in the rectal cavity to release the drug.

Formulations suitable for parenteral administration conveniently comprise a sterile aqueous

preparation of the active compound, which is preferably isotonic with the blood of the recipient.

Nasal spray formulations comprise purified aqueous solutions of the active compound with preservative agents and isotonic agents. Such formulations are preferably adjusted to a pH and isotonic state compatible with the nasal mucous membranes.

Formulations for rectal administration may be presented as a suppository with a suitable carrier such as cocoa butter, or hydrogenated fats or hydrogenated fatty carboxylic acids.

Ophthalmic formulations are prepared by a similar method to the nasal spray, except that the pH and isotonic factors are preferably adjusted to match that of the eye.

Topical formulations comprise the active compound dissolved or suspended in one or more media such as mineral oil, petroleum, polyhydroxy alcohols or other bases used for topical pharmaceutical formulations. The addition of other accessory ingredients, vide infra, may be desirable.

Since individual subjects may present a wide variation in severity of symptoms and each drug has its unique therapeutic characteristics, it is up to the practitioner to determine a subject's response to treatment and vary the dosages accordingly.

Typical daily oral dose, in general, for the reduction of multidrug resistance lies within the range of from about 0.5 μg to about 10 mg per kg body weight and, preferably within the range of from 50 μg to 1 mg per kg body weight and can be administered up to four times daily. The daily IV dose for the reversal of multidrug resistance lies within the range of from about 1μg to about 10 mg per

kg body weight and, preferably, within the range of from 10μg to 500 μg per kg body weight.

For the treatment of multidrug resistant tumors, the compounds of the present invention may be utilized to sensitize multidrug resistant tumor cells to chemotherapeutic agents and also to reduce the effective dosage of a chemotherapeutic agent during the course of treatment. For these purposes, the compounds of this invention may be utilized with one or more chemotherapeutic agents which are useful in treating cancer selected from the group consisting of: vinblastine, vincristine, dactinomycin, daunorubicin, doxorubicin, mithramycin, bleomycin, actinomycin D, etoposide, teniposide and mitomycin-C. The compounds of the present invention may be administered in combination with, in conjunction with, prior to or concurrent to the administration of chemotherapeutic agents.

For example, the compounds of the present invention can be given in combination with such compounds as: vinblastine, vincristine, dactinomycin, daunorubicin, doxorubicin, actinomycin D, etoposide and mithramycin, or combinations of such compounds, or salt or other derivative forms thereof.

The weight ratio of a compound of the present invention to chemotherapeutic agent or compound may vary and will depend upon the effective dose of each ingredient. Generally, an effective dose of each ingredient (i.e., chemotherapeutic agent and MDR-reducing compound) will be used. Thus, for example, when a compound of the present invention is combined with vinblastine, vincristine, dactinomycin, daunorubicin, doxorubicin or mithramycin, the weight ratio of the compound of the present invention to vinblastine, vincristine, dactinomycin, daunorubicin, doxorubicin or mithramycin, ranges from about 1000:1 to

about 1:1000, preferably about 100:1 to 1:100. Combinations of a compound of the present invention and an anticancer agent or compound will generally also be within the aforementioned range, but in each case, an effective dose of each active ingredient should be used.

Tumors which can be treated by the method of this invention include both benign and malignant tumors or neoplasms, and include melanomas, lymphomas, leukemias, and sarcomas. Illustrative examples of tumors are cutaneous tumors, such as malignant melanomas and mycosis fungoides; hematologic tumors such as leukemias, for example, acute lymphoblastic, acute myelocytic or chronic myelocytic leukemia; lymphomas, such as Hodgkin's disease or malignant lymphoma; gynecologic tumors, such as ovarian and uterine tumors; urologic tumors, such as those of the prostate, bladder or testis; soft tissue sarcomas, osseus or non-osseus sarcomas, breast tumors; tumors of the pituitary, thyroid and adrenal cortex; gastrointestinal tumors, such as those of the esophagus, stomach, intestine and colon; pancreatic and hepatic tumors; laryngeae papillomestasas and lung tumors. Of course those tumors which typically are or become multidrug resistant are most beneficially treated with the method of this invention. Such tumors include colon tumors, lung tumors, stomach tumors, and liver tumors. The effective amount of chemotherapeutic agent used in the method of this invention varies widely and depends on factors such as the patient, the tumor tissue type and its size, and the particular chemotherapeutic agent selected. The amount is any effective amount and can be readily determined by those skilled in the art.

A preferred category of multiple drug resistant tumor cells to be treated by the method of the present invention is multiple drug resistant cells characterized by the multidrug transporter - mediated pumping of

antineoplastic agents out of the tumor cells. The multidrug transporter protein is described in M. Gottesman and I. Pastan, J. Biol. Chem.263:12163 (1988). Thus, tumor cells treated by the present invention are preferably those characterized by (a) the expression of the multidrug transporter protein at high levels, or (b) the ability to express the multidrug transporter protein upon exposure to an antineoplastic agent.

Exemplary tumor cells which express the multidrug transporter at high levels (intrinsically resistant cells) are adenocarcinoma cells, pancreatic tumor cells, carcinoid tumor cells, chronic myelogenous leukemia cells in blast crisis, and non-small cell lung carcinoma cells.

Exemplary tumor cells having the ability to express the multidrug transporter protein upon exposure to an antineoplastic agent are neuroblastoma cells, pheochromocytoma cells, adult acute lymphocytic leukemia cells, adult acute nonlymphocytic leukemia cells, nodular poorly differentiated lymphoma cells, breast cancer cells and ovarian cancer cells.

A preferred group of tumor cells for treatment in the present invention are the adenocarcinomas, including adenocarcinomas of adrenal, kidney, liver, small intestine and colon tissue, with kidney adenocarcinoma cells particularly preferred.

Preferred antineoplastic agents for use in the present invention are those which induce multidrug resistance in cells. Exemplary of such antineoplastic agents are vinca alkaloids, epipodophyllotoxins, anthracycline antibiotics, actinomycin D, plicamycin, puromycin, gramicidin D, taxol, colchicine, cytochalasin B, emetine, maytansine, and amsacrine (or "mAMSA"). Preferred

are vinca alkaloids, epipodophyllotoxins, anthracycline antibiotics, actinomycin D, and plicamycin.

The vinca alkaloid class is described in Goodman and Gilman's The Pharmacological basis of Therapeutics, 1277-1280 (7th ed. 1985) (hereafter "Goodman and Gilman"). Exemplary of vinca alkaloids are vincristine, vinblastine, and vindesine.

The epipodophyllotoxin class is described in Goodman and Gilman, supra at 1280-1281. Exemplary of epipodophyllotoxins are etoposide, etoposide orthoquinone, and teniposide.

The anthracycline antibiotic class is described in Goodman and Gilman, supra at 1283-1285. Exemplary of anthracycline antibiotics are daunorubicin, doxorubicin, mitoxantraone, and bisanthrene. Daunorubicin and doxorubicin are preferred.

Actinomycin D, also called Dactinomycin, is described in Goodman and Gilman, supra at 1281-1283. Plicamycin, also called mithramycin, is described in Goodman and Gilman, supra at 1287-1288.

In accordance with another embodiment of the present invention, there is provided a method of enhancing the intracellular accumulation of a molecule within a cell. Presently preferred molecules for transport by this method are those molecules which do not naturally occur in such cell, but which are capable of entering such cell (such as, for example, chemotherapeutic agents as described herein). This method comprises

a) contacting the cell with a sufficient concentration of the above-described MDR-reducing compounds, to inhibit extracellular transport of the molecule from the cell; and

b) contacting the resulting cell with said molecule so as to effect intracellular accumulation of said molecule within the cell.

In accordance with the practice of this invention, molecules for which one might seek to enhance intracellular accumulation include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, coltricin, doxirubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoid, procaine, tetracaine, lidocaine, propraolol, puromycin, and the like.

As used herein the term "enhancing the intracellular accumulation of a molecule" means increasing the intracellular level of the molecule in the cell by preventing the transport of the molecule from the cell by the P-glycoprotein efflux pump.

Preferred cell types for treatment in accordance with this embodiment of the present invention include tumor cells, especially neural tumor cells.

In accordance with yet another embodiment of the present invention, there is provided a method of enabling a molecule to cross the blood-brain barrier and accumulate in the central nervous system, which molecule is not normally capable of crossing such barrier and accumulating in the central nervous system. This method comprises

a) contacting the blood brain barrier with a sufficient concentration of the above-described MDR-reducing compounds to enable said molecule to cross the barrier; and

b) contacting the resulting barrier with said molecule so as to enable said molecule to cross the barrier.

The disruption of genes encoding the transport proteins which impart MDR has recently been found to facilitate the transport of molecules across the blood-brain barrier (see, for example, Schinkel et al., in Cell 77:491-502 (1994). Thus, the use of MDR-reducing compounds, which have been shown herein to block the effects of the transport proteins which cause MDR, are indicated to facilitate the transport of molecules across the blood-brain barrier.

In accordance with yet another aspect of the present invention the use of the above-described compounds, or pharmaceutically acceptable salts thereof, for a variety of applications is provided, such as, for example, in the manufacture of a medicament for enhancing the therapeutic effect of an antineoplastic agent, for the manufacture of a medicament for inhibiting multiple drug resistance in tumors, for the manufacture of a medicament for increasing the sensitivity of a resistant tumor to an antineoplastic agent and/or for the manufacture of a medicament for selectively inhibiting the growth of tumor cells.

The invention will now be described in greater detail by reference to the following non-limiting examples.

Example 1
Cell Staining Method to Determine Cell Growth

Equal numbers of cells of an anchorage dependent mammalian cell line are seeded in growth medium (e.g., alpha MEM plus 10% FBS) into a suitable culture vessel, e.g., plastic 96 well tissue culture plates. A cytotoxic drug (e.g. doxorubicin) is added to the cells in the dishes at various concentrations, typically ranging between 0 and 100 μM.

Following about 72 hours of continuous exposure to the cytotoxic agent, the growth medium is decanted and the cells are washed with a suitable buffer, e.g., phosphate buffered saline (PBS). About 2 ml of a solution of 2% methylene blue (methylene blue is dissolved in a solution of about 50% ethanol in water) is added to the cells on the dishes. The dye is allowed to contact the cells for about 2 minutes. Excess dye is washed away with cold water and the plates are air dried. The dye stained cells are then solubilized by adding an equal volume of a solution of a detergent, e.g., 1% N-lauroyl-sarcosine, to all wells.

The amount of dye remaining in the wells directly correlates with the number of cells in the well. The amount of methylene blue dye in the wells can be measured spectrophotometrically by measuring absorbance at 600 nm using an electronic ELISA plate spectrophotometer (Minireader II, Dynatech Laboratories, Alexandria, VA). Typical results show decreased absorbance at 600 nm with increasing cytotoxic drug concentration, indicating increased cell death with increased drug concentration.

Example 2
Visual Assay of Multidrug Resistance

Multidrug resistance has been detected *in vitro* in single cell suspensions and in cell monolayers. Yoshimura et al., Cancer Letters Vol. 50:45 (1990) used the accumulation of rhodamine dye to screen for agents that overcome multidrug resistance in a cell line ("reversing agents"). The dye is accumulated in multidrug-resistant cells at a lower rate than it is accumulated in non-resistant cells. Thus multidrug-resistant cells can be distinguished from non-resistant cells by comparing intracellular dye levels.

Dye levels in multidrug-resistant cells are then monitored in the presence and absence of verapamil, a known chemosensitizer (reversing agent used in chemotherapy to facilitate the uptake of a chemotherapeutic drug in drug-resistant tumor cells). It is typically found that the dye accumulates to normal levels when the multidrug resistance phenotype is reversed with verapamil. The dye is administered to cells in a confluent monolayer. The cells are then either washed, solubilized, and the dye detected with a fluorescence spectrometer, or scanned in microtitre wells with a fluorescence microplate reader.

Etferth et al., Arzneim-Forsch vol. 38:1171 (1988) have also developed an *in vitro* assay to detect the multidrug resistance phenotype. They describe comparing the levels of rhodamine dye in a cell sample with the levels of dye found in a control sample of normal cells. The dye is detected by forming a single cell suspension, pipetting the suspension onto slides, administering the dye to the cells on the slide, and detecting dye uptake of cells on the slide.

Herweijer et al., Invest New Drugs Vol. 7:442 (1989) describe the use of on-line flow cytometry to detect cells with the multidrug resistance phenotype in a single cell suspension. The accumulation kinetics of a fluorescent drug are measured on line, first in the absence, and then in the presence of a reversing agent.

Konen et al., J. Histochem. Cytochem. Vol. 37:1141 (1989), describe assaying efflux activity of the multidrug resistance transport system using fluorescence microscopy to monitor the accumulation of drugs in single cultured cells that were transformed with multidrug resistance DNA. They showed that the efflux pathway was inhibited when the cells were incubated with verapamil.

Example 3

Effects of RU486 and Puromycin on the Viability of Cells Displaying MDR Phenotype

Cortisol, dexamethasone, aldosterone and other similar steroids appear to be substrates for transport by P-glycoprotein. In contrast, steroids such as progesterone and cortexolone are not transported. However, progesterone has been shown to bind to the mouse and human P-glycoproteins and to inhibit their function, even though it is not transported [see, for example, Yang et al., J. Biol. Chem. 264:782 (1989); Qian & Beck, J. Biol. Chem. 265:18753 (1990); or Yang et al., J. Biol. Chem. 265:10282 (1990)]. RU38486 (RU486) was developed as an antiprogestin with high affinity binding to both the progesterone and glucocorticoid receptors [See, for example, Gravanis et al., J. Clin. Endocrinol. Metab. 60:156 (1985) or Tentsch et al., J. Steroid Biochem. 31:549 (1988)]. Its structure contains an 11ß-(dimethyl aminophenyl) substitution, which contributes to its antagonist properties.

If RU486 can inhibit P-glycoprotein function, it was speculated that it might be able to reverse the drug resistant phenotype of the murine thymoma cell line S7CD-5. The S7CD-5 cell line was derived from a steroid-sensitive mouse line, WEHI-7 [See Bourgeois et al., Mol. Endocrinol. 7:840 (1993) and Johnson et al., Cancer Res. 44:2435 (1984)]. It expresses the *mdr1* gene and is resistant to a variety of drugs including colchicine, puromycin, daunomycin and dexamethasone. The resistance to dexamethasone is due to reduced intracellular accumulation of steroid even though the glucocorticoid receptors in S7CD-5 are present at normal levels and are fully functional. The drug resistance in this cell line is effectively reversed by 5 μM verapamil, an established inhibitor of P-glycoprotein function [se, for example, Ford and Hait, Pharmacol. Rev. 42:155 (1990); Willingham et al.,

Cancer Res. 46:5941 (1986); or Sofa et al., J. Biol. Chem. 262:7884 (1987)].

When separate cultures of S7CD-5 cells (initially containing 4 x 10^4 cells/ml) were incubated with no additions, 5μM RU486, 20 μM puromycin, or 5μM RU486 plus 20 μM puromycin, the following observations were made. At the times indicated, samples were removed and the concentration of viable cells evaluated using a trypan blue exclusion test. The values represent an average of 2 determinations for each time point. Neither drug, by itself, has a significant effect on the viability or proliferation of the cells. The combination, on the other hand, causes a complete loss of viability within 42 hours. The result with RU486 alone is not unexpected since it normally has little or no agonist activity for the glucocorticoid receptor [see Bourgeois et al., EMBO J. 3:751 (1984)]. These results are consistent with the possibility that RU486 acts at another target and promotes the accumulation of puromycin in the cells.

Example 4
Reversal of Puromycin Resistance in Cells Displaying MDR Phenotype

The relative abilities of verapamil and RU486 to alter the resistance of S7CD-5 to puromycin are compared. Separate sets of S7CD-5 cultures initially containing 5 x 10^4 cells/ml were incubated in the indicated concentrations of puromycin for 5 days. The cultures contained: no additions, -0-; 5 μM RU486, -Δ-; or 5 μM verapamil, -▲-. Another set of cultures containing the parental W7TB line without additions, -•-, is shown for comparison.

At the end of the incubation period, the turbidities of the cultures (A_{660}) were measured and normalized to values from the cultures without puromycin.

These values reflect the amount of proliferation in the cultures and each is the average of duplicate determinations [see Johnson et al., supra]. As shown in Figure 1, the two drugs are seen to have a very similar effect. Each lowers the puromycin resistance approximately 17-fold, to a level nearly equal to that seen with the sensitive parental cell line.

Example 5
Reversal of Daunomycin Resistance in Cells Displaying MDR Phenotype

Separate sets of S7CD-5 cultures, containing RU486 or verapamil, were set up and incubated in the indicated concentrations of daunomycin (see Figure 2) in a manner similar to the experiment described in Example 4. A set of cultures containing W7TB cells was again used for comparison.

The results presented in Figure 2 demonstrate that RU486 and verapamil also have the capacity to completely reverse the resistance to daunomycin seen in the S7CD-5 cells. RU486 and verapamil have the additional capacity to reverse colchicine resistance in S7CD-5 cells and in cells expressing the *mdr3* gene. This similarity in behavior between RU486 and verapamil is particularly significant since verapamil has been found to be one of the most potent inhibitors of P-glycoprotein function.

Example 6
Concentration Dependence of Drug Resistance Reversal

To determine the relative concentrations at which RU486 and verapamil begin to reverse drug resistance the following experiments were carried out. The effects of growing S7CD-5 cells in increasing concentrations of either verapamil or RU486 in the presence or absence of 20 μM

puromycin were determined. Without puromycin, verapamil and RU486 have only modest effects on the cells proliferation and no visible effect on viability. In the presence of puromycin, verapamil causes a sharp decrease in proliferation at concentrations above 0.5 μM. RU486 exhibits a very similar profile, only slightly displaced to higher concentrations. Indeed, microscopic inspection of the cultures revealed that all of the cells were dead in the presence of 20 μM puromycin and of verapamil or RU486 at concentrations above 2 μM.

Example 7

Efflux of Rhodamine 123 from Cells Displaying MDR Phenotype

Previous studies have shown that P-glycoproteins transport the fluorescent drug rhodamine 123 [see, for example, Neyfakh, Exp. Cell Res. 174:168 (1988) or Chaudhary and Roninson, Cell 66:85 (1991)]. Rhodamine presents a comparison by flow cytometry of the capacities of RU486 and verapamil to inhibit the efflux of rhodamine 123.

S7CD-5 cells were incubated for 30 minutes in 500 ng/ml of rhodamine 123. At the end of the incubation, the cells were placed into fresh medium without rhodamine and maintained at 0°C until analysis. Samples of cells were isolated by centrifugation and resuspended in phosphate buffered saline at 37°C containing the drugs indicated in Figure 3. The controls contained no drug. Flow cytometric assays were performed on a Becton Dickinson (BD) FACStar Plus cell sorter and subsequent listmode data analysis done on a Sun SPARCStation 2 with software facilitating the time slicing of kinetics assay. Laser excitation was at 100 mW from an Argon laser tuned to 477 nm. The flow rate was maintained between 100 to 200 cells per second and rhodamine 123 fluorescence measured over a 4 decade range through the standard BD FL1 fluorescein bandpass filter

(520 nm). All listmode data files were collected on a Hewlett Packard Consort 32 System using LYSYS II (BD) configured to include time as a correlated listmode parameter over a period of 1024 seconds. Typically, files were time sliced into 32 points and rhodamine 123 fluorescence averaged over a period of 4 seconds per point. The averaged values (geometric means) for each time slice were then normalized to the first point in the series to yield a value representing the percent rhodamine 123 remaining within the cells for each time point.

In the absence of drug, 35% of the rhodamine was retained after 4.5 minutes at 37°C, and RU486 at 1 μM had no effect on this rate. In the presence of 5 μM and 10 μM RU486 the rates of efflux were significantly slower, with 35% of the rhodamine being retained after 8 and 14 minutes respectively. In comparison, it took 16.5 minutes to efflux the same amount of rhodamine 123 in the presence of 5 μM verapamil. The change with 5 μM RU486 is 0.8 fold (4.5 minutes to 8 minutes), the change with 10 μM RU486 is 2.1 fold (4.5 minutes to 14 minutes), while the change with 5 μM verapamil is 2.7 fold (4.5 minutes to 16.5 minutes). Therefore, the comparison suggests that RU486 is at least one third as effective as verapamil in inhibiting rhodamine efflux.

The ability of RU486 to inhibit drug efflux is compared with that of dexamethasone and progesterone, with the results shown in Figure 4. In this experiment, 10 μM dexamethasone had no influence on the efflux rate. As expected, 10 μM progesterone had a measurable effect, but it was considerably smaller than the inhibition caused by 5 μM RU486. At these hormone concentrations, RU486 was more than three times as effective as progesterone at inhibiting drug efflux.

These studies demonstrate that RU486 has the capacity to efficiently inhibit drug efflux promoted by the mouse *mdr1* P-glycoprotein and to reverse the multidrug resistant phenotype conveyed by expression of this protein. RU486 can achieve this effect at relatively low doses, just above 1 μM are readily achievable [see, for example, Heikinheimo and Kekkonen, Ann. Med. 25:71 (1993)]. Thus, given the similarity between the mouse and human P-glycoproteins, these results support the proposed use of RU486 as a clinically-effective and useful chemosensitizing agent. This is particularly true since RU486 does not have the considerably toxic side effects associated with other chemosensitizing compounds such as the calcium-channel blocker verapamil.

Example 8
Effect of Various Chemosensitizers on the Vinblastine Sensitivity of Human Leukemic Cells

Four separate cultures of CEM/VBL_{100} cells (an MDR variant of an human CEM leukemic cell line, selected as resistant to the chemotherapeutic agent, vinblastine) were prepared containing ~$5x10^4$ cells/ml. Each culture, with either no drug addition (designated by □); or containing 5μM verapamil (designated by ▲); or containing 5μM RU486 (designated by ■); or containing 5μM progesterone (designated by Δ) was contacted with varying amounts of vinblastine (as shown in Figure 5). Thus, cells were dispensed into multiwell plates (1 ml/well) and the indicated concentration of vinblastine was added. The cultures were incubated for 8 days at 37°C in the presence of 5% CO_2. At the end of the incubation period, the turbidity (at 660 nm) of the resulting cell suspension was measured in each well, then normalized relative to the value obtained from cultures without any added vinblastine. The resulting measurements evaluate the proliferative

capacity of the cells. Each point in Figure 5 represents the average of two separate determinations.

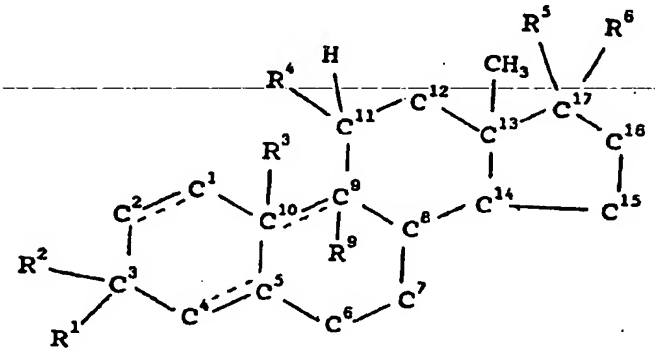
As can be seen upon inspection of the results presented in Figure 5, RU486 is nearly as effective as verapamil in facilitating the transport of vinblastine into otherwise vinblastine resistant cells. Even progesterone shows a small improvement in the ability of vinblastine to effect the viability of otherwise vinblastine resistant cells.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

That which is claimed is:

1. A method for reducing multidrug resistance in a subject undergoing chemotherapy, said method comprising:

administering to said subject, in conjunction with a chemotherapeutic agent, an effective amount of a compound having the structure:



wherein:

$R^1 = R^2 = O$; or R^1 = hydrogen and

R^2 is OR, wherein R is selected from hydrogen, lower alkyl or trimethylsilyl; and

R^3 is absent when there is a double bond between C^9 and C^{10}, or when there is an epoxide bridging C^9 and C^{10}, or R^3 is hydrogen or methyl; and

R^4 is -OR', wherein R' is lower alkyl or trimethylsilyl, or R^4 is an organic radical having in the range of 4 up to 18 carbon atoms containing at least one atom selected from the group consisting of oxygen, nitrogen, phosphorus and silicon, wherein the atom immediately adjacent C^{11} is carbon, and said organic radical includes a cyclic moiety selected from an alicyclic ring, a heterocyclic ring, a carbocyclic aromatic ring, or a heterocyclic aromatic ring, wherein said cyclic moiety contains an

oxygen-, nitrogen-, phosphorus- or silicon-bearing substituent,

R^5 is hydrogen or OR, wherein R is as defined above, or R^5 is a 3, 4 or 5 atom bridging species which forms part of a 3-, 5-, 6- or 7-membered ring including C^{16} and C^{17} as part of the ring; and

R^6 is selected from:

$-C(O)-CH_3$,

$-CH(OH)-CH_3$,

$-C(O)-CH_2OH$, or

$-(R^7)_{0,1}-R^8$, wherein

R^7, when present, is a saturated or unsaturated hydrocarbyl radical having in the range of 1 up to 8 carbon atoms, and

R^8 is selected from hydrogen, halogen, trimethylsilyl, phenyl or substituted phenyl, and

R^9 is absent when there is a double bond between C^9 and C^{10}, or, when present, R^9 is selected from hydrogen or halogen.

2. A method according to claim 1 wherein R^4 is

$-(CH_2)_x-(C_6H_{3,4})-[X_{0,1}-(CH_2)_y-X']_z$;

wherein:

x is 0-3,

y is 0-2,

z is 1 when the aromatic ring is C_6H_4, or 2 when the aromatic ring is C_6H_3,

X is O or S,

X' is hydrogen, OR, NR''_2, $N^+R''_3$, $\overset{O^-}{\underset{|}{N^+}}R''_2$, or NO_2, wherein R is as defined above and R'' is hydrogen or a lower alkyl group.

3. A method according to claim 2 wherein x is 0.

4. A method according to claim 2 wherein z is 1, and the substituent on the phenyl ring is located at the para position of the ring.

5. A method according to claim 4 wherein the substituent on the phenyl ring is selected from:

$-O-CH_2CH_2-NR"_2$,

$-O-CH_2CH_2-N^+R"_3$, or

$-O-CH_2CH_2-N^+(O^-)R"_2$.

6. A method according to claim 2 wherein z is 2, and the substituents on the phenyl ring are located at the meta and para positions of the ring.

7. A method according to claim 5 wherein the para substituent is selected from OR, $NR"_2$, $N^+R"_3$, $N^+(O^-)R"_2$, or NO_2.

8. A method according to claim 1 wherein R^5 is a 3, 4 or 5 atom bridging species selected from alkylene, or O-, C(O)-, N-, and/or S-containing alkylene moiety.

9. A method according to claim 8 wherein said bridging species is selected from $-(CH_2)_{3-5}-$, $-C(CH_3)_2-$, $-O-(CH_2)_{2-4}-$, $-O-(CH_2)_{1-3}-O-$, $-(CH_2)_{1,2}-O-(CH_2)_{1,2}-$ or $-O-CH_2-C(O)-CH_2-O-$.

10. A method according to claim 1 wherein R^7 is selected from $-(CH_2)_x-CR'=CR'-(CH_2)_x-$ or $-(CH_2)_x-C\equiv C-(CH_2)_x-$, wherein each x is independently selected from 0-6.

11. A method according to claim 1 wherein R^8 is selected from hydrogen, chloro, trimethylsilyl or phenyl.

12. A method according to claim 1 wherein R^9 is hydrogen.

13. A method according to claim 1 wherein R^6 is selected from:

$-C(O)-CH_3$,
$-CH(OH)-CH_3$, or
$-C(O)-CH_2OH$.

14. A method according to claim 1 wherein R^6 has the structure:

$-(CH_2)_{0,1}-C\equiv C-R^8$, wherein
R^8 is selected from hydrogen, chloro, trimethylsilyl or phenyl.

15. A method according to claim 1 wherein R^6 is selected from

$-CH_2-CH=CH_2$,
$-CH_2-C\equiv CH$,
$-C\equiv C-H$,
$-C\equiv C-CH_3$,
$-C\equiv C-Cl$,
$-C\equiv C-C_6H_5$, or
$-C\equiv C-SiMe_3$.

16. A method according to claim 1 wherein said compound contains a double bond between C^1 and C^2.

17. A method according to claim 16 wherein said compound contains a double bond between C^4 and C^5.

18. A method according to claim 17 wherein said compound contains a double bond between C^9 and C^{10}.

19. A method according to claim 16 wherein said compound contains a double bond between C^9 and C^{10}.

20. A method according to claim 1 wherein said compound contains a double bond between C^4 and C^5.

21. A method according to claim 20 wherein said compound contains a double bond between C^9 and C^{10}.

22. A method according to claim 1 wherein said compound contains a double bond between C^9 and C^{10}.

23. A method according to claim 1 wherein said compound has an epoxide bond bridging C^9 and C^{10}.

24. A method according to claim 16 wherein said compound has an epoxide bond bridging C^9 and C^{10}.

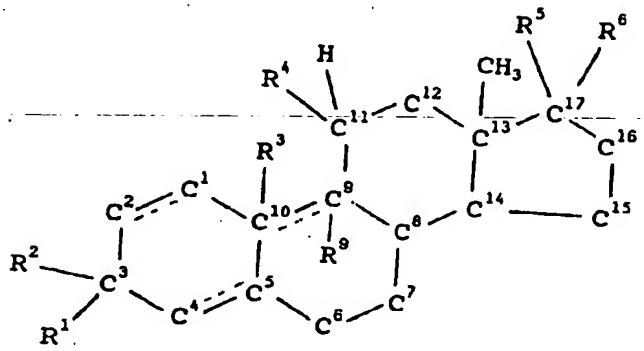
25. A method according to claim 17 wherein said compound has an epoxide bond bridging C^9 and C^{10}.

26. A method according to claim 20 wherein said compound has an epoxide bond bridging C^9 and C^{10}.

27. A method according to claim 1 wherein
$R^1 = R^2 = O$,
R^3 is methyl,
R^4 is $-C_6H_4-NMe_2$ or $-C_6H_4-\overset{O^-}{\underset{|}{N^+}}R'_2$,
R^5 is OH,
R^6 is selected from:
$-CH_2-CH=CH_2$,
$-CH_2-C\equiv CH$,
$-C\equiv C-H$,
$-C\equiv C-CH_3$,
$-C\equiv C-Cl$,
$-C\equiv C-C_6H_5$, or
$-C\equiv C-SiMe_3$.

28. A method of enhancing the intracellular accumulation of a molecule within a cell, said method comprising:

(a) contacting the cell with a sufficient concentration of a compound having the structure:



wherein:

$R^1 = R^2 = O$; or R^1 = hydrogen and

R^2 is OR, wherein R is selected from hydrogen, lower alkyl or trimethylsilyl; and

R^3 is absent when there is a double bond between C^9 and C^{10}, or when there is an epoxide bridging C^9 and C^{10}, or R^3 is hydrogen or methyl; and

R^4 is $-OR'$, wherein R' is lower alkyl or trimethylsilyl, or R^4 is an organic radical having in the range of 4 up to 18 carbon atoms containing at least one atom selected from the group consisting of oxygen, nitrogen, phosphorus and silicon, wherein the atom immediately adjacent C^{11} is carbon, and said organic radical includes a cyclic moiety selected from an alicyclic ring, a heterocyclic ring, a carbocyclic aromatic ring, or a heterocyclic aromatic ring, wherein said cyclic moiety contains an

oxygen-, nitrogen-, phosphorus- or silicon-bearing substituent,

R^5 is hydrogen or OR, wherein R is as defined above, or R^5 is a 3, 4 or 5 atom bridging species which forms part of a 3-, 5-, 6- or 7-membered ring including C^{16} and C^{17} as part of the ring; and

R^6 is selected from:

$-C(O)-CH_3$,

$-CH(OH)-CH_3$,

$-C(O)-CH_2OH$, or

$-(R^7)_{0,1}-R^8$, wherein

R^7, when present, is a saturated or unsaturated hydrocarbyl radical having in the range of 1 up to 8 carbon atoms, and

R^8 is selected from hydrogen, halogen, trimethylsilyl, phenyl or substituted phenyl, and

R^9 is absent when there is a double bond between C^9 and C^{10}, or, when present, R^9 is selected from hydrogen or halogen

to inhibit extracellular transport of the molecule from the cell; and

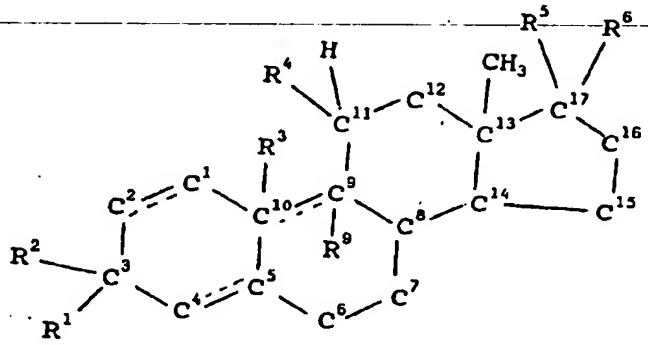
(b) contacting the resulting cell with said molecule so as to effect intracellular accumulation of said molecule within the cell.

29. A method according to claim 28 wherein the cell is a tumor cell.

30. A method according to claim 29 wherein said tumor cell is a neural cell.

31. A method of enabling a molecule to cross the blood-brain barrier and accumulate in the central nervous system, which molecule is not normally capable of crossing such barrier and accumulating in the central nervous system, said method comprising:

(a) contacting the blood-brain barrier with a sufficient concentration of a compound having the structure:



wherein:

$R^1 = R^2 = O$; or R^1 = hydrogen and

R^2 is OR, wherein R is selected from hydrogen, lower alkyl or trimethylsilyl; and

R^3 is absent when there is a double bond between C^9 and C^{10}, or when there is an epoxide bridging C^9 and C^{10}, or R^3 is hydrogen or methyl; and

R^4 is -OR', wherein R' is lower alkyl or trimethylsilyl, or R^4 is an organic radical having in the range of 4 up to 18 carbon atoms containing at least one atom selected from the group consisting of oxygen, nitrogen, phosphorus and silicon, wherein the atom immediately adjacent C^{11} is carbon, and said organic radical includes a cyclic moiety selected from an alicyclic ring, a heterocyclic ring, a carbocyclic aromatic ring, or a heterocyclic aromatic ring,

wherein said cyclic moiety contains an oxygen-, nitrogen-, phosphorus- or silicon-bearing substituent,

R^5 is hydrogen or OR, wherein R is as defined above, or R^5 is a 3, 4 or 5 atom bridging species which forms part of a 3-, 5-, 6- or 7-membered ring including C^{16} and C^{17} as part of the ring; and

R^6 is selected from:

$-C(O)-CH_3$,

$-CH(OH)-CH_3$,

$-C(O)-CH_2OH$, or

$-(R^7)_{0,1}-R^8$, wherein

R^7, when present, is a saturated or unsaturated hydrocarbyl radical having in the range of 1 up to 8 carbon atoms, and

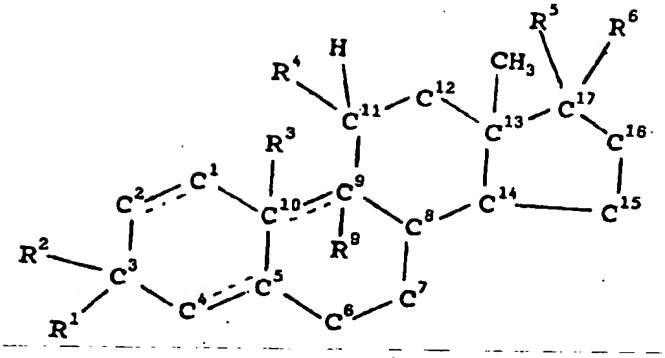
R^8 is selected from hydrogen, halogen, trimethylsilyl, phenyl or substituted phenyl, and

R^9 is absent when there is a double bond between C^9 and C^{10}, or, when present, R^9 is selected from hydrogen or halogen

to enable said molecule to cross the barrier, and

(b) contacting the resulting barrier with said molecule so as to enable said molecule to cross the barrier.

32. Use of a compound having the structure:



wherein:

R^1 = R^2 = O; or R^1 = hydrogen and

R^2 is OR, wherein R is selected from hydrogen, lower alkyl or trimethylsilyl; and

R^3 is absent when there is a double bond between C^9 and C^{10}, or when there is an epoxide bridging C^9 and C^{10}, or R^3 is hydrogen or methyl; and

R^4 is -OR', wherein R' is lower alkyl or trimethylsilyl, or R^4 is an organic radical having in the range of 4 up to 18 carbon atoms containing at least one atom selected from the group consisting of oxygen, nitrogen, phosphorus and silicon, wherein the atom immediately adjacent C^{11} is carbon, and said organic radical includes a cyclic moiety selected from an alicyclic ring, a heterocyclic ring, a carbocyclic aromatic ring, or a heterocyclic aromatic ring, wherein said cyclic moiety contains an oxygen-, nitrogen-, phosphorus- or silicon-bearing substituent,

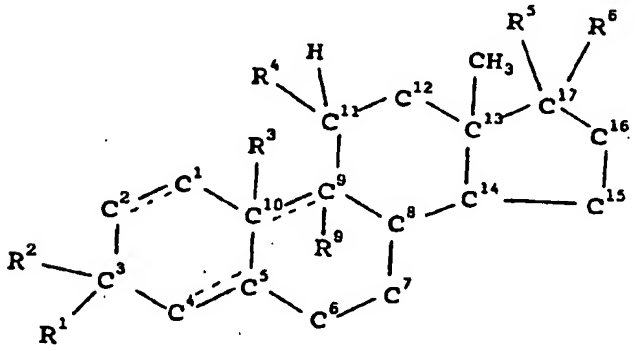
R^5 is hydrogen or OR, wherein R is as defined above, or R^5 is a 3, 4 or 5 atom bridging species which forms part of a 3-, 5-, 6- or 7-membered ring including C^{16} and C^{17} as part of the ring; and

R^6 is selected from:

$-C(O)-CH_3$,
$-CH(OH)-CH_3$,
$-C(O)-CH_2OH$, or
$-(R^7)_{0,1}-R^8$, wherein
R^7, when present, is a saturated or unsaturated hydrocarbyl radical having in the range of 1 up to 8 carbon atoms, and
R^8 is selected from hydrogen, halogen, trimethylsilyl, phenyl or substituted phenyl, and
R^9 is absent when there is a double bond between C^9 and C^{10}, or, when present, R^9 is selected from hydrogen or halogen

or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for enhancing the therapeutic effect of an antineoplastic agent.

33. A pharmaceutical composition comprising



wherein:

$R^1 = R^2 = O$; or R^1 = hydrogen and
R^2 is OR, wherein R is selected from hydrogen, lower alkyl or trimethylsilyl; and
R^3 is absent when there is a double bond between C^9 and C^{10}, or when there is an epoxide

bridging C^9 and C^{10}, or R^3 is hydrogen or methyl; and

R^4 is -OR', wherein R' is lower alkyl or trimethylsilyl, or R^4 is an organic radical having in the range of 4 up to 18 carbon atoms containing at least one atom selected from the group consisting of oxygen, nitrogen, phosphorus and silicon, wherein the atom immediately adjacent C^{11} is carbon, and said organic radical includes a cyclic moiety selected from an alicyclic ring, a heterocyclic ring, a carbocyclic aromatic ring, or a heterocyclic aromatic ring, wherein said cyclic moiety contains an oxygen-, nitrogen-, phosphorus- or silicon-bearing substituent,

R^5 is hydrogen or OR, wherein R is as defined above, or R^5 is a 3, 4 or 5 atom bridging species which forms part of a 3-, 5-, 6- or 7-membered ring including C^{16} and C^{17} as part of the ring; and

R^6 is selected from:

$-C(O)-CH_3$,

$-CH(OH)-CH_3$,

$-C(O)-CH_2OH$, or

$-(R^7)_{0,1}-R^8$, wherein

R^7, when present, is a saturated or unsaturated hydrocarbyl radical having in the range of 1 up to 8 carbon atoms, and

R^8 is selected from hydrogen, halogen, trimethylsilyl, phenyl or substituted phenyl, and

R^9 is absent when there is a double bond between C^9 and C^{10}, or, when present, R^9 is selected from hydrogen or halogen

in the form of a pharmaceutically acceptable non-toxic acid addition salt.

34. A pharmaceutical composition according to claim 33 wherein said composition is solid, semi-solid or liquid.

FIG. 1

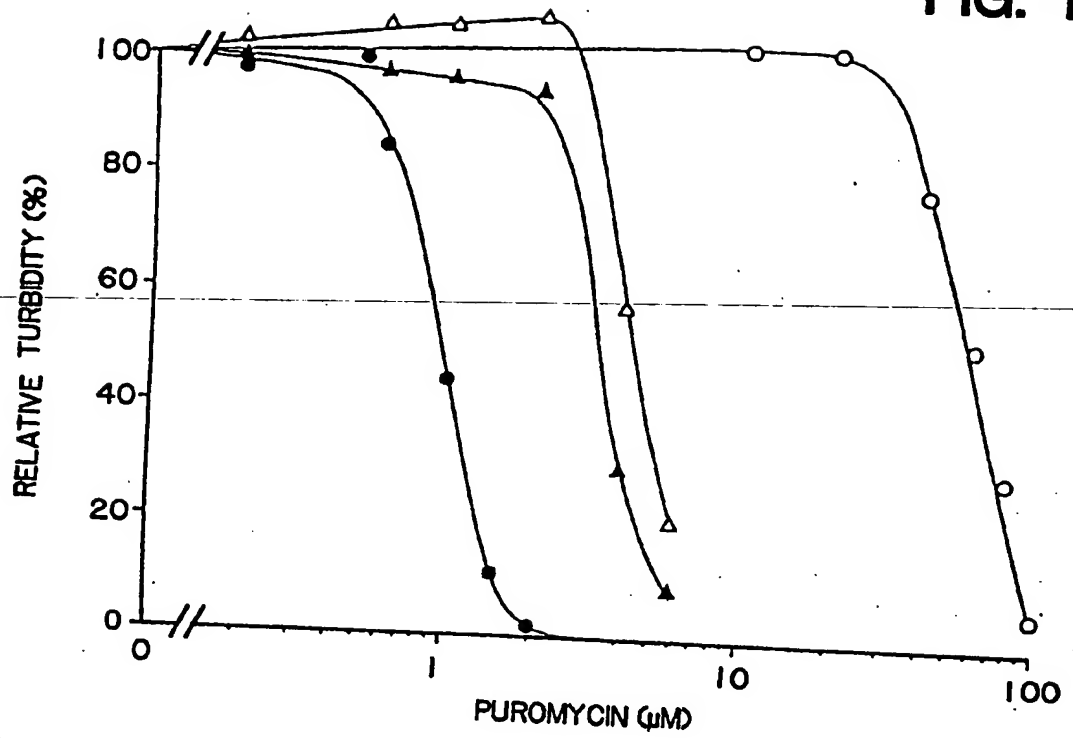
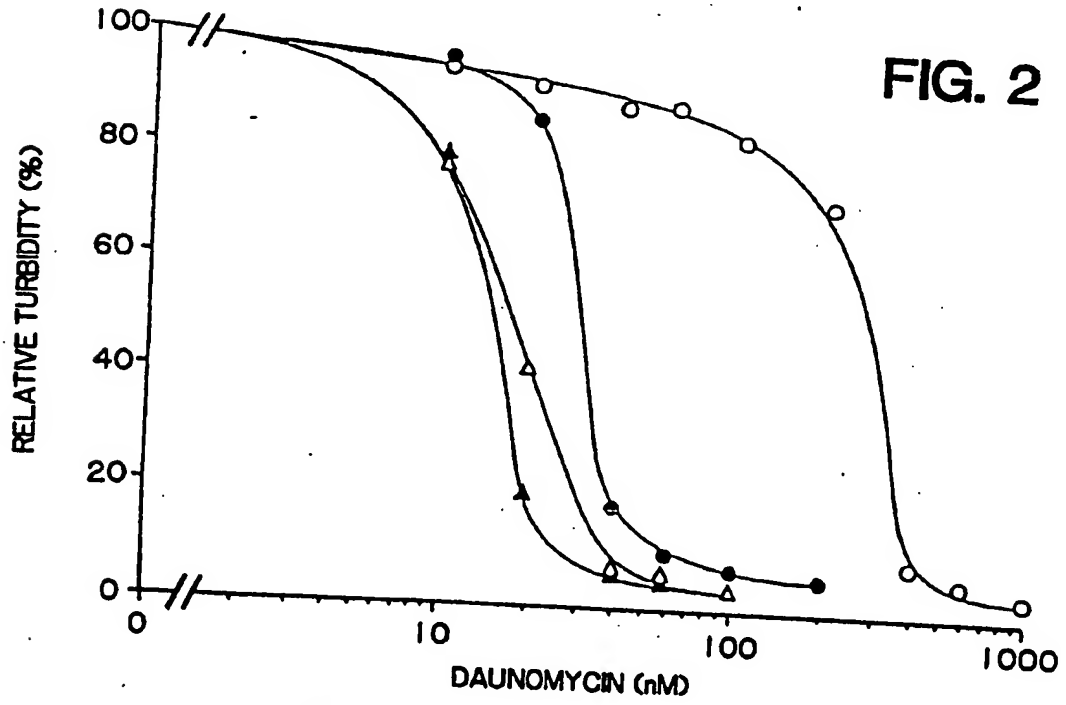

RELATIVE TURBIDITY (%)
100
80
60
40
20
0
0
1
10
100
PUROMYCIN (μM)

FIG. 2


RELATIVE TURBIDITY (%)
100
80
60
40
20
0
0
10
100
1000
DAUNOMYCIN (nM)

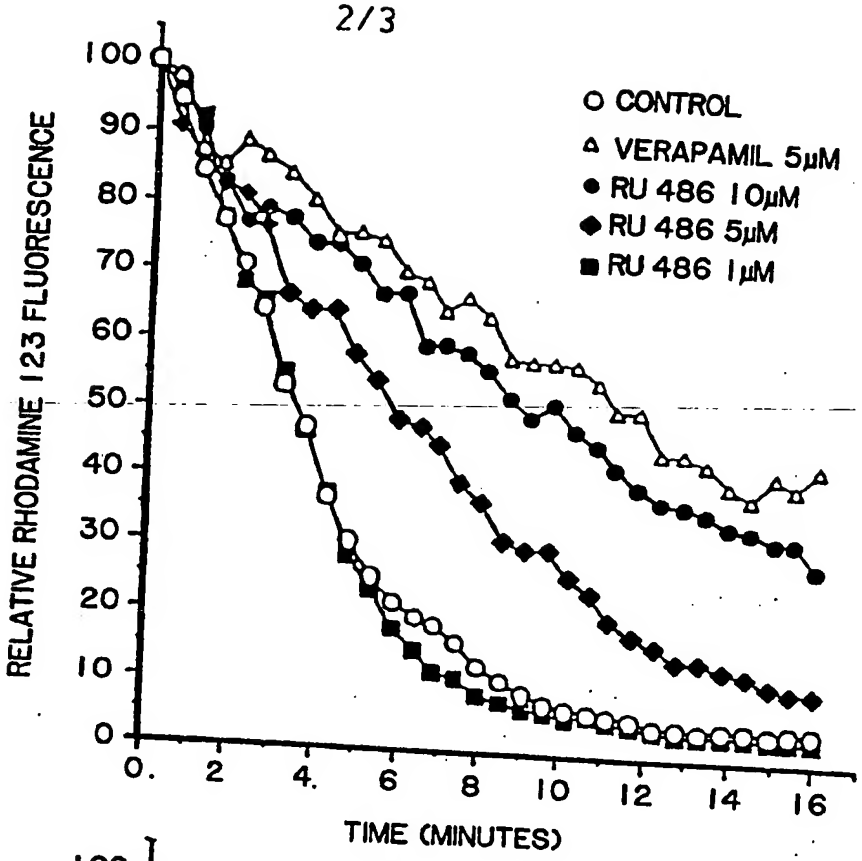

RELATIVE RHODAMINE 123 FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
0.
2
4.
6
8
10
12
14
16
TIME (MINUTES)
CONTROL
VERAPAMIL 5μM
RU 486 10μM
RU 486 5μM
RU 486 1μM

FIG. 3

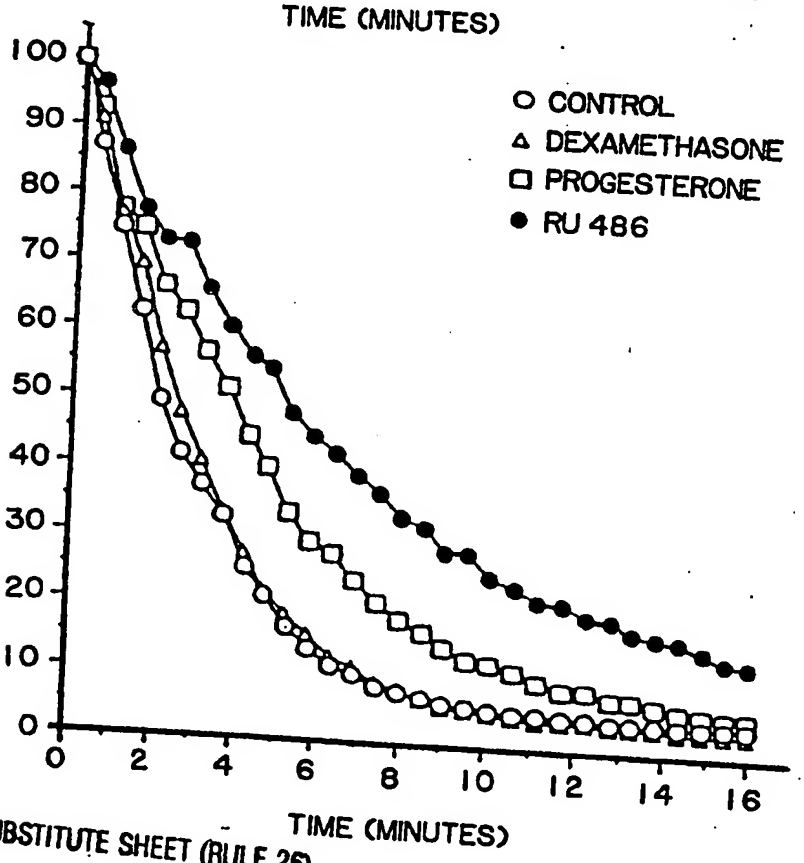

100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
TIME (MINUTES)
CONTROL
DEXAMETHASONE
PROGESTERONE
RU 486

FIG. 4

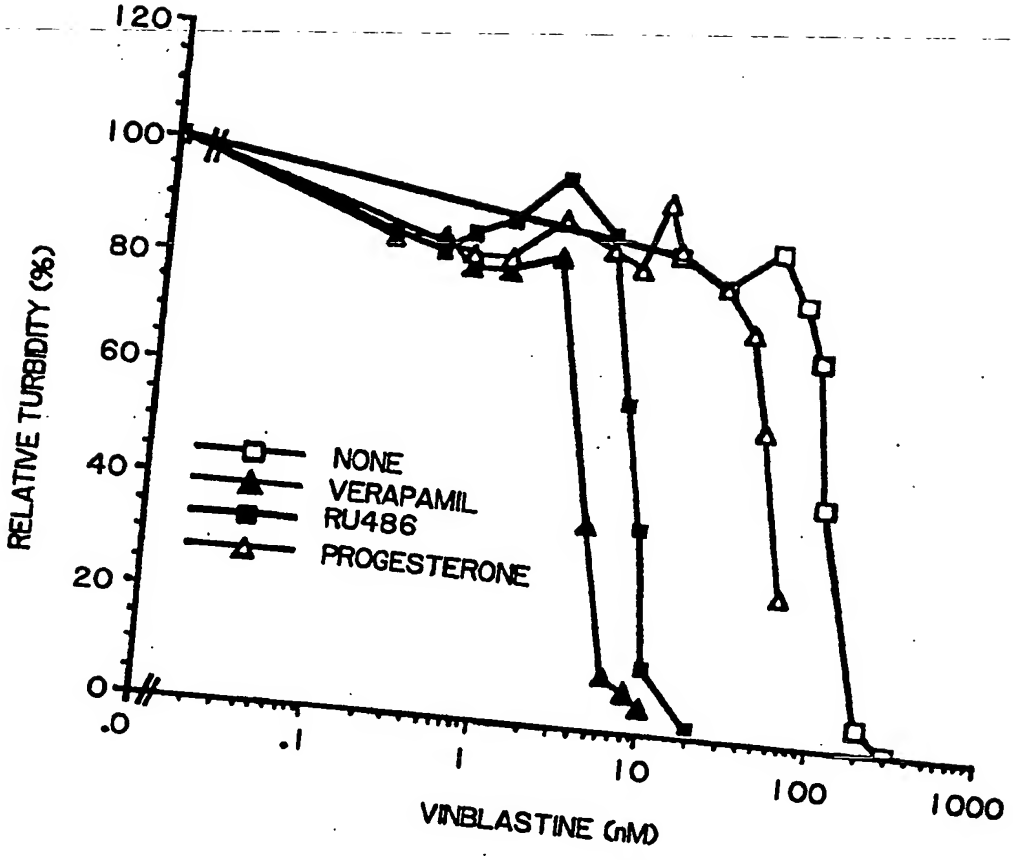

120
100
80
60
40
20
0
RELATIVE TURBIDITY (%)
NONE
VERAPAMIL
RU486
PROGESTERONE
.0
.1
1
10
100
1000
VINBLASTINE (nM)

FIG. 5

INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/US94/14624

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 31/56
US CL :514/179,169,171,172,173,174,175,176,177,178,179
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/179,169,171,172,173,174,175,176,177,178,179

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,386,085 (Teutsch et al) 31 May 1983, column 1, line 40.	1-27, 33-34

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
29 MARCH 1995

Date of mailing of the international search report
27 APR 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
K. MACMILLAN/W.JARVIS
Telephone No. (703) -308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Int .national application No.
PCT/US94/14624

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-27, and 33-34

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

In .national application No.
PCT/US94/14624

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-27, and 33-34, drawn to a method and composition for reducing multidrug resistance.

Group II, claim(s) 28-30, drawn to a method for enhancing intracellular accrual of a molecule.

Group III, claim(s) 31-33, drawn to method of enabling a molecule to cross the blood-brain barrier.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: A reference which anticipates one of Groups I-III would not render the other obvious absent ancillary art. 37 CFR 1.475(d).